

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
23 September 2004 (23.09.2004)

PCT

(10) International Publication Number
WO 2004/081235 A1

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number:
PCT/US2004/002946

(22) International Filing Date: 2 February 2004 (02.02.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/384,497 7 March 2003 (07.03.2003) US

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AI, AG, AI, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Euro-
pean (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR,
GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NI, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 2004/081235 A1

(54) Title: NUCLEIC ACID BIOSENSORS

(57) Abstract: Sensors comprising aptazymes capable of detecting the presence and concentration of effectors, as well as methods
of using such sensors, are disclosed.

NUCLEIC ACID BIOSENSORS

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The subject matter of this application may in part have been funded by the Department of Energy (DEFG02-01ER63179) and the National Science Foundation (DMR-0117792). The government may have certain rights in this invention

BACKGROUND

Long considered strictly genetic material, DNA was shown in 1994 to be able to act as an enzyme (Breaker and Joyce 1994). Like RNAzymes, DNAzymes can catalyze nucleic acid and phosphoramidate bond cleavage, ligation, phosphorylation, and porphyrin metallation (Lu 2002). Because of their stability and catalytic capabilities, DNAzymes promise to be important in a large array of applications (Lu 2002).

Aptamers are nucleic acids (such as DNA or RNA) that recognize targets with high affinity and specificity (Ellington and Szostak 1990, Jayasena 1999). Aptazymes (also called allosteric DNA/RNAzymes or allosteric (deoxy)ribozymes) are DNA/RNAzymes regulated by an effector (the target molecule). They typically contain an aptamer domain that recognizes an effector and a catalytic domain (Hesselberth et al. 2000, Soukup and Breaker 2000, Tang and Breaker 1997). The effector can either decrease or increase the catalytic activity of the aptazyme through specific interactions between the aptamer domain and the catalytic domain. Therefore, the activity of the aptazyme can be used to monitor the presence and quantity of the effector. This strategy has been used to select and design aptazyme sensors for diagnostic and sensing purposes (Breaker 2002, Robertson and Ellington 1999, Seetharaman et al. 2001). DNA aptazymes are the most attractive candidate for sensor development because DNA is much less expensive to synthesize and more stable than RNA. In addition, general strategies to design DNA aptazymes, by introducing aptamer motifs close to the catalytic core of DNAzymes, are available (Wang et al. 2002). High cleavage activity requires the presence of effector molecules that upon binding to the aptamer motif, can allosterically modulate the activity of the catalytic core part of the aptazyme.

In vitro selection methods can be used to obtain aptamers for a wide range of target molecules with exceptionally high affinity, having dissociation constants as high

as in the picomolar range (Brody and Gold 2000, Jayasena 1999, Wilson and Szostak 1999). For example, aptamers have been developed to recognize metal ions such as Zn(II) (Ciesiolka et al. 1995) and Ni(II) (Hofmann et al. 1997); nucleotides such as adenosine triphosphate (ATP) (Huizenga and Szostak 1995); and guanine (Kiga et al. 1998); co-factors such as NAD (Kiga et al. 1998) and flavin (Lauhon and Szostak 1995); antibiotics such as viomycin (Wallis et al. 1997) and streptomycin (Wallace and Schroeder 1998); proteins such as HIV reverse transcriptase (Chaloin et al. 2002) and hepatitis C virus RNA-dependent RNA polymerase (Biroccio et al. 2002); toxins such as cholera whole toxin and staphylococcal enterotoxin B (Bruno and Kiel 2002) and bacterial spores such as the anthrax (Bruno and Kiel 1999). Compared to antibodies, DNA/RNA based aptamers are easier to obtain and less expensive to produce because they are obtained *in vitro* in short time periods (days vs. months) and with limited cost. In addition, DNA/RNA aptamers can be denatured and renatured many times without losing their biorecognition ability. These unique properties make aptamers an idea platform for designing highly sensitive and selective biosensors (Hesselberth et al. 2000).

Radioisotope and fluorescence signals are often used to detect aptamer and aptazyme activity. Radioisotope-labeling has the advantage of minimal perturbation for the binding ability of aptamers and aptazymes (Rusconi et al. 2002, Seetharaman et al. 2001); however, safety and disposal concerns prevent this method from broad use. Fluorescence provides significant signal amplification and enables real-time monitoring of concentration fluctuations. However, determining effective parameters for using fluorophores is inefficient, requiring trial and error. If too close to the binding site, fluorophores may prevent the effector from binding; if too remote, no signal will be detected. To overcome this difficulty when using aptamers, fluorophores are incorporated into nucleotides during aptamer selection (Jhaveri et al. 2000). Many fluorophores are easily photo-bleached.

A powerful alternative to fluorophore and radio-isotope detection is colorimetry (Cao et al. 2001, Rakow and Suslick 2000, Smith et al. 1999). Colorimetric detection minimizes detection costs and safety concerns, and is well suited for on-site and real-time detection. In a colorimetric cocaine sensor based on aptamers, cocaine displaces a dye in the binding site of a cocaine aptamer (Stojanovic and Landry 2002). Because the dye has different absorption properties when bound to the aptamer, the presence of

cocaine is indicated by a color change. However, finding an appropriate dye for a particular aptamer requires screening a large number of dyes. Moreover, the extinction coefficient for organic dyes seldom exceeds $10^6 \text{ L} \cdot \text{mole}^{-1} \cdot \text{cm}^{-1}$, necessitating high dye concentration for simple visual observation.

- 5 Metallic particles have extinction coefficients three orders of magnitude higher than those of organic dyes (Link et al. 1999). For effective detection, they may be used in low concentrations (nanomolar) for use as detection agents with aptamers.

SUMMARY

- 10 In a first aspect, the invention is drawn to a sensor system for detecting an effector, having a nucleic acid enzyme (comprising an aptamer comprising a binding site for the effector), a substrate for the nucleic acid enzyme and particles having a second polynucleotide that is at least partially complementary to the substrate. The enzyme may be DNA, the effector may activate or inhibit the enzyme, the particles may be gold
15 particles or other metal colloids or polystyrene latex particles. The effector may be adenosine, anthrax, an anthrax-derived molecule, small pox, a small pox-derived molecule, HIV, an HIV-derived molecule, an antibiotic or cocaine. The nucleic acid enzyme may be both SEQ ID NOS:5 and 6, both SEQ ID NOS:8 and 9 or SEQ ID NO:1; the substrate may be SEQ ID NOS:4, 7 or 2. The sensor system may be mixed with a
20 sample to detect an effector. Mg(II) and Pb(II) may also be added to the sample. When detecting an effector, a step of heating the sensor system to disrupt pairing between the polynucleotides may also be included. The presence of an effector is indicated by a color change in the sample, or of aggregated particles, which may precipitate in the sample.

- 25 In a second aspect, the invention is drawn to a sensor system for detecting an effector, having a nucleic acid enzyme of DNA (comprising an aptamer comprising a binding site for the effector), a substrate for the nucleic acid enzyme, gold particles having a second polynucleotide that is at least partially complementary to the substrate, and Mg(II). The sensor system may further comprise Pb(II). This sensor system may be
30 used to detect an effector by mixing it with a sample.

 In a third aspect, the invention is drawn to methods of detecting an effector, where the ingredients of a sample, a nucleic acid enzyme (having an aptamer comprising a binding site for the effector), a substrate for the nucleic acid enzyme, and particles

having a polynucleotide that is at least partially complementary to the substrate. The ingredients may be mixed in different sequences. The nucleic acid enzyme may be both SEQ ID NOS:5 and 6, both SEQ ID NOS:8 and 9 or SEQ ID NO:1; the substrate may be SEQ ID NOS:4, 7 or 2.

- 5 In a fourth aspect, the invention is drawn to kits for detecting an effector, a sensor system for detecting an effector, having a nucleic acid enzyme (comprising an aptamer comprising a binding site for the effector), a substrate for the nucleic acid enzyme and particles having a second polynucleotide that is at least partially complementary to the substrate. The particles may be gold particles or other metal colloids or polystyrene latex
10 particles. The effector may be adenosine, anthrax, an anthrax-derived molecule, small pox, a small pox-derived molecule, HIV, an HIV-derived molecule, an antibiotic or cocaine. The nucleic acid enzyme may be both SEQ ID NOS:5 and 6, both SEQ ID NOS:8 and 9 or SEQ ID NO:1; the substrate may be SEQ ID NOS:4, 7 or 2. The sensor system may be mixed with a sample to detect an effector. Mg(II) and Pb(II) may also be
15 added to the sample. The presence of an effector is indicated by a color change in the sample, or of aggregated particles, which may precipitate in the sample.

- The kit may be supplied such that the substrate, particles and nucleic acid enzyme are supplied in separate containers or the substrate, particles and nucleic acid enzyme are supplied in a single container. Furthermore, the kit may be supplied such that the
20 substrate, particles and nucleic acid enzyme are supplied as an aggregate. The kit may further include control solutions, such as a solution having a known concentration of an effector. A color chart, wherein the colors indicate a concentration of the effector, may also be included to facilitate quantification.

25 DESCRIPTION OF THE FIGURES

Figure 1 shows an example of a nucleic acid enzyme and its substrate.

Figure 2 shows an adenosine aptazyme; rA indicates a single adenosine ribonucleotide.

- Figure 3 shows a schematic representation of the colorimetric detection of
30 adenosine. The aptazyme system can act as linker for DNA attached gold particles to form aggregations, which have a blue color (reaction A). In the presence of adenosine and metal ions, the substrate can be cleaved (reaction B). The cleaved substrate can no longer act as linker for particles and the color remains red (reaction C).

Figure 4 shows the extinction spectra of separated 13 nm diameter gold particles (solid line) and gold particles aggregation linked by aptazymes (dashed line) in the visible region. Aggregation induced by DNA linkers shifts the peak of the surface plasmon band from 522 nm to 540 nm.

5 Figure 5 shows the melting curve of the aptazyme assembled gold particles in 25 mM Tris-acetate buffer, pH 7.2, 300 mM NaCl.

Figure 6 shows the relationship between the substrate quantity and the degree of aggregation of gold particles. The vertical axis is the ratio of the extinction between 522 nm and 700 nm. The arrow indicates 6 pmol of substrate.

10 Figure 7 shows the kinetics of color change of the aptazyme-gold particle sensor.

Figure 8 shows the quantification of adenosine concentration using UV-vis spectroscopy.

15 DETAILED DESCRIPTION

The present invention makes use of the discovery that the cleavage of a nucleic acid substrate by an aptazyme upon binding of an effector can be detected colorimetrically. In the presence of the effector, the substrate is cleaved and particles attached to polynucleotides that can hybridize to the substrate strand are dispersed,
20 resulting in a color change. This system combines the benefit of elements that can recognize any molecule of choice with high sensitivity and ease-of-use provided by colorimetric detection.

The system comprises at least three parts:

- (1) a nucleic acid-based enzyme having an effector-binding site (such as in
25 aptamers) and a co-factor such as metal ions that catalyze substrate cleavage;
- (2) a nucleic acid substrate for the nucleic acid-based enzyme, wherein interior portions of the substrate sequence is complementary to portions of the enzyme sequence; and
- (3) particles attached to polynucleotides that are complementary to the 3'-
30 and 5'- termini of the substrate.

To detect the target effector, the complementary portions of the polynucleotides (the polynucleotides attached to the particles complementary to the 3'- and 5'-termini of the substrate strand, and the 5'- and 3'-termini of the nucleic acid-based enzyme

complementary to interior substrate strand sequences) are annealed in the presence of a sample suspected of containing the targeted effector. If the effector is absent, the aptazyme is either inactive or shows substantially reduced activity, resulting in no or little substrate cleavage and thus aggregation of the particles. If the effector is present, the enzyme is active and cleaves the substrate, preventing aggregate formation because the link between the particles is broken by the enzymatic cleavage step. In the case of gold particles, the aggregated state displays a blue color, while the dispersed state (or the non-aggregate state) is red in color. The presence of the target analyte as an effector can be detected based on the appearance of the color of the sensor system. More importantly, the concentration or the amount of the target analyte as an effector can be quantified by the degree of color deviation from blue or red. For example, a low concentration of the effector will result in a small percentage of substrate cleavage, small percentage of particles in the non-aggregate state, small deviation from the blue color and thus purple color can be observed. On the other hand, a high concentration of the effector will result in a large percentage of substrate cleavage, large percentage of particles in the non-aggregate state, large deviation from the blue color and thus pink/red can be observed. For a more quantitative analysis, spectrometry such as the extinction ratio between 522 nm and 700 nm can be used. The analyte as an effector may also decrease or inhibit the aptazyme activity instead of increase the aptazyme activity. In that case, the effect and color changes are the opposite to what described above. The effect may also be detected by other methods, such as aggregate precipitation. If the effector is absent, no cleavage will occur.

By replacing the aptamer domain with aptamers recognizing pre-selected effectors, colorimetric sensors for any desired effector can be easily made and used.

Definitions

An "effector" is a molecule that, when bound to an enzyme having an effector binding site, can enhance or inhibit enzyme catalysis. An "effector binding site" may be "specific," that is, binding only one effector molecule in the presence of other effector molecules. An example of effector binding site specificity is when only Zn(II) ions bind in the presence of many other ions, such as Mn(II), Mg(II) or Pb(II). Alternatively, an effector binding site may be "partially" specific (binding only a class of molecules), or "non-specific" (having molecular promiscuity). Examples of effectors include metal

ions, anthrax, anthrax-derived molecules, small pox or small pox-derived molecules, pollutants (such as nitrogen fertilizers, toxic molecules, *etc.*), cocaine, human immunodeficiency virus (HIV) and HIV-derived molecules.

A "nucleic acid-based enzyme" is an enzyme that principally contains nucleic acids, such as ribozymes (RNAzymes), deoxyribozymes (DNAzymes), and aptazymes. Nucleic acids may be natural, unnatural or modified nucleic acids. Peptide nucleic acids (PNAs) are also included. A nucleic acid-based enzyme requires a metal "co-factor" for efficient substrate cleavage and/or specific effector binding. Common co-factors include Mg(II) and Pb(II).

"Polynucleotide" refers to a nucleic acid sequence having at least two or more nucleotides. Polynucleotides may contain naturally-occurring nucleotides and modified nucleotides. PNA molecules are also embraced by this term.

"Sensitivity" refers to the limits of detection of a analytical device. In the context of the aptazyme-based sensors of the invention, sensitivity refers to the least concentration and highest concentration of an effector that the sensor can detect.

"Base-pairing" refers to the ability of a polynucleotide to form at least one hydrogen bond with a nucleotide under low stringency conditions. The nucleotide may be in a second polynucleotide or to a nucleotide found within the first polynucleotide. A polynucleotide is partially complementary to a second polynucleotide when the first polynucleotide is capable of forming at least one hydrogen bond with the second polynucleotide. To be partially complementary, a polynucleotide may have regions wherein base pairs may not form surrounded by those regions that do, form loops, stem-loops, and other secondary structures.

A nucleic acid-based enzyme having an effector (or effectors) binding site

A number of nucleic acid enzymes have been discovered or developed, having diverse catalytic activities (Tables 1 and 2). For catalytic function, the enzymes usually depend on one or more ion co-factors. *In vitro* selection may be used to "enhance" selectivity and sensitivity for a particular ion. Nucleic acid enzymes that catalyze molecular association (ligation, phosphorylation, and amide bond formation) or dissociation (cleavage or transfer) are particularly useful for the methods and compositions of the invention.

- A nucleic acid enzyme that catalyzes the cleavage of a nucleic acid in the presence of an effector is used. The nucleic acid enzyme may be RNA (ribozyme), DNA (deoxyribozyme), a DNA/RNA hybrid enzyme, or a peptide nucleic acid (PNA) enzyme. PNAs comprise a polyamide backbone and naturally-occurring nucleoside bases
- 5 (available from, e.g., Biosearch, Inc. (Bedford, MA)). Ribozymes that may be used include group I and group II introns, the RNA component of the bacterial ribonuclease P, hammerhead, hairpin, hepatitis delta virus and *Neurospora* VS ribozymes. Also included are *in vitro* selected ribozymes, such as those previously isolated (Tang and Breaker 2000). Ribozymes tend to be less stable than deoxyribozymes; thus
- 10 deoxyribozyme are preferred. Deoxyribozymes with extended chemical functionality are also desirable (Santoro *et al.*, 2000).

Table 1 Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.

Reaction	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat}/k_{uncat} ^a	Reference
<i>Phosphoester centers</i>				
Cleavage	0.1	0.03	10 ⁵	(Vaish et al. 1998)
Transfer	0.3	0.02	10 ¹³	(Tsang and Joyce 1996)
Ligation	100	9	10 ⁹	(Ekland et al. 1995)
Phosphorylation	0.3	40	>10 ⁵	(Lorsch and Szostak 1994)
Mononucleotide polymerization	0.3	5000	>10 ⁷	(Ekland and Bartel 1996)
<i>Carbon centers</i>				
Aminoacylation	1	9000	10 ⁶	(Illangasekare and Yarus 1997)
Aminoacyl ester hydrolysis	0.02	0.5	10	(Piccirilli et al. 1992)
Aminoacyl transfer	0.2	0.05	10 ³	(Lohse and Szostak 1996)
N-alkylation	0.6	1000	10 ⁷	(Wilson and Szostak 1995)
S-alkylation	4 × 10 ³	370	10 ³	(Wecker et al. 1996)
Amide bond cleavage	1 × 10 ⁵		10 ²	(Dai et al. 1995)
Amide bond formation	0.04	2	10 ⁵	(Wiegand et al. 1997)
Peptide bond formation	0.05	200	10 ⁶	(Zhang and Cech 1997)
Diels-Alder cycloaddition	>0.1	>500	10 ³	(Tarasow et al. 1997)
<i>Others</i>				
Biphenyl isomerization	3 × 10 ⁵	500	10 ²	(Prudent et al. 1994)
Porphyrin metallation	0.9	10	10 ³	(Conn et al. 1996)

^aReactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments. k_{cat}/k_{uncat} is the rate enhancement over uncatalyzed reaction.

Table 2 Deoxyribozymes isolated through *in vitro* selection.

Reaction	Cofactor	$k_{\max}(\text{min})^b$	$k_{\text{cat}}/k_{\text{uncat}}$	Reference
RNA transesterification	Pb^{2+}	1	10^3	(Breaker and Joyce 1994)
	Mg^{2+}	0.01	10^5	(Breaker et al. 1995)
	Ca^{2+}	0.08	10^5	(Faulhammer and Famulok 1997)
	Mg^{2+}	10	$>10^5$	(Santoro and Joyce 1997)
DNA cleavage	None	0.01	10^8	(Geyer and Sen 1997)
	L-histidine	0.2	10^6	(Roth and Breaker 1998)
	Zn^{2+}	~40	$>10^5$	(Li et al. 2000)
	Cu^{2+}	0.2	$>10^6$	(Carmi et al. 1996)
DNA ligation	Cu^{2+} or Zn^{2+}	0.07	10^5	(Cuenoud and Szostak 1995)
DNA phosphorylation	Ca^{2+}	0.01	10^9	(Li and Breaker 1999)
5',5'-pyrophosphate formation	Cu^{2+}	5×10^{-2}	$>10^{10}$	(Li et al. 2000)
Porphyrin metalation	None	1.3	10^3	(Li and Sen 1996)

^b k_{\max} is the maximal rate constant obtained under optimized conditions.

Methods of producing ribozymes and deoxyribozymes include chemical oligonucleotide synthesis, polymerase chain reaction (PCR), DNA cloning and replication, or any other methods in the art. Preferably the nucleic acid enzymes are DNA/RNA hybrids and PNAs. Nucleotides containing modified bases, phosphates, or sugars may also be used; in some instances, these modified nucleotides may be advantageous for stability or confer effector specificity. Examples of modified bases include inosine, nebularine, 2-aminopurine riboside, N⁷-deazaadenosine, and O⁶-methylguanosine (Earnshaw and Gait 1998). Modified sugars and phosphates include 2'-deoxynucleoside, abasic, propyl, phosphorothioate, and 2'-O-allyl nucleoside (Earnshaw and Gait 1998).

A nucleic acid enzyme that cleaves a nucleic acid strand separate from the strand comprising the enzyme is a *trans*-acting enzyme. Using *trans*-acting enzymes allows for multiple rounds of substrate cleavages, since the enzymatic product is removed. An example of such a nucleic acid enzyme is 17E (SEQ ID NO:1); the corresponding substrate is 17DS (SEQ ID NO:2; r denotes a single ribonucleotide); both are presented in Table 3A and illustrated in Figure 1. Other examples are also given in Table 3B.

Table 3A DNA enzymes and substrates

Molecule	SEQ ID NO:	Sequence ^c	# of nucleotides
Enzyme (17E)	1	catctcttct ccgagccggt cgaaatagtg agt	33
Substrate for 17E (17DS)	2	actcactatr ggaagagatg	21
Enzyme: JLYL1 "8-17" half	5	tctcttctcc gagccggtcg aaatattgga ggaagctc	38
ATP half	6	gagctggagg aaaaagtgag tc	22
Sustrate for JLYL1	4	gactcactat rggaagaga	19
Enzyme: JLYL2 "8-17" half	8	cacgagttga catctcttct ccgagccggt cgaaatattg gaggaagctc	50
ATP half	9	gagctggagg aaaaagtgag tctcacagat gagt	34
Substrate for JLYL2	7	actcatctgt gagactcact atrggaagag atgtcaactc gtg	43
^c "r" denotes a single ribonucleotide, such as adenosine ribonucleotide			

Table 3B RNA/DNA based aptamers and RNA/DNAzymes

RNA/DNA based aptamers and their targets ⁴		RNA/DNAzymes ^{5, 6-10}
Organic dyes ^{11,12}	Xanthene ³⁹	8-17 DNAzyme ¹³⁻¹⁵
Theophyllin ¹⁶	Kanamycin A ⁶⁰	10-23 DNAzymes ^{13,17}
Dopamine ¹⁸	Lividomycin ⁶⁰	Hammerhead ^{19,20}
Hoechst 33258 ²¹	Tobramycin ⁶¹	Hairpin ^{19,22}
Sulforhodamine B ^{23,24}	Neomycin B ^{62,63}	Leadzyme ²⁵
Cellobiose ²⁶	Viomycin ⁶⁴	Hepatitis Delta Virus ^{27,28}
D-tryptophan ²⁹	Chloramphenicol ⁶⁵	Group I Intron ^{30,31}
L-arginine ³²⁻³⁷	Streptomycin ⁶⁶	Spliceosome ³⁸
L-citrullin ^{32,36}	HIV-1 Rev peptide ^{67,68}	Ribosome ³⁹
L-argininamide ⁴⁰	Vasopressin ⁶⁹	DNA nuclease activity ⁴¹
L-valine ⁴²	Spectinomycin ⁷⁰	Ligase activity ⁴³
L-isoleucine ⁴⁴	L-tyrosinamide ⁷¹	Kinase activity ⁴⁵
AMP/ATP ⁴⁶⁻⁵⁰	HIV-1 RNase H ⁷²	Phosphoramidate bond cleavage ⁵¹
Guanosine ⁵²	Chitin ⁷³	Porphyrin metallation ⁵³
FMN ^{47,54}	Human Thrombin ⁷⁴	Peroxidase activity ⁵⁵
NAD ⁵⁴	cAMP ⁷⁵	
Vitamin B ₁₂ ⁵⁶	Cholic acid ⁷⁶	
8-oxo-dG ⁵⁷	Hematoporphyrin ⁷⁷	
5'-cap ⁵⁸	HIV-1 Tat/Zn ^{2+,78}	
	Anthrax spores ⁷⁹	

Directed mutagenesis can be used to change one or more properties of a nucleic acid enzyme or its substrate. Using 17E and 17DS as an example, one may wish to alter the avidity of the two arms of the hybridized enzyme and substrate. The "arms" are those areas displaying Watson-Crick base-pairing in Figure 1. To alter avidity, the length of the arms is changed. Increasing the length of the arms increases the number of Watson-Crick bonds, thus increasing avidity; decreasing the length decreases avidity. Decreasing the avidity of

the arms facilitates the removal of substrate from the enzyme, thus allowing for faster enzymatic turnover.

Another method of decreasing avidity includes creating mismatches between the enzyme and the substrate. Alternatively, the G-C content of the arms may be altered. The effect of any directed change should be monitored to ensure that the enzyme retains the desired activity, including ion sensitivity and selectivity. For example, to ensure that the mutated enzyme maintains sensitivity and selectivity for adenosine, one would test to determine if the mutated enzyme remained reactive in the presence of adenosine (sensitivity) and maintained its lower level of activity in the presence of other effectors (selectivity).

In vitro selection of aptamers

Aptamers and aptazymes that bind a desired effector can be isolated by *in vitro* selection. *In vitro* selection is a technique in which RNA or DNA molecules with certain functions are isolated from a large number of sequence variants through multiple cycles of selection and amplification (Chapman and Szostak 1994, Joyce 1994). DNAzymes and RNAzymes with maximized activities or novel catalytic abilities, as well as aptamers can be obtained using, for example, the technique of systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990).

In vitro selection is typically initiated with a large collection (pool) of randomized sequences, usually containing 10^{13} - 10^{15} sequence variants. Chemical synthesis of a set of degenerated polynucleotides using standard phosphoramidite chemistry can be used to generate such randomized pools. The 3'-phosphoramidite compounds of the four nucleosides (adenosine, cytosine, guanine, thymidine) are premixed and used to synthesize the polynucleotides; randomness is generated by controlling the ratio of the four phosphoroamidites. Biases can also be achieved, as well as holding a phosphoramidite constant at a specific position. Other strategies for creating randomized DNA libraries include mutagenic polymerase chain reaction (PCR) and template-directed mutagenesis (Cadwell and Joyce 1992, Cadwell and Joyce 1994, Tsang and Joyce 1996). If *in vitro* selection of RNA molecules is desired, randomized DNA libraries are first converted to an RNA library by *in vitro* transcription.

The randomized libraries are then screened for molecules possessing a desired function, such as binding the targeted effector, and are isolated. Separation may be achieved using affinity column chromatography (using, *e.g.*, the targeted effector), gel electrophoresis, or selective amplification of a tagged reaction intermediate. The selected molecules are amplified, using, for example, PCR for DNA, or isothermal amplification reaction for RNA. These selected, amplified molecules are then mutated (reintroducing diversity) using, for example, mutagenic PCR to attempt to select for molecules with yet higher activity. These three steps, selection, amplification and mutation, are repeated, often with increasing selection stringency, until sequences with the desired activity dominate the pool.

Novel nucleic acid enzymes isolated from random sequences *in vitro* have extended the catalytic repertoire of RNA and DNA (Table 1). Deoxyribozymes catalyze fewer types of reactions compared to ribozymes (Table 2). The catalytic rate (k_{cat}) of most deoxyribozymes is comparable to that of ribozymes catalyzing the same reaction. In certain cases, the catalytic efficiency (k_{cat}/K_m) of nucleic acid enzymes even exceeds protein enzyme catalytic efficiency.

In vitro selection can be used to change the ion specificity or binding affinity of existing nucleic acid enzymes, or to obtain nucleic acid enzymes specific for desired substrates. For example, the Mg^{2+} concentration required for optimal hammerhead ribozyme activity has been lowered using *in vitro* selection to improve the enzyme performance under physiological conditions (Conaty et al. 1999, Zillmann et al. 1997).

Often nucleic acid enzymes developed for a specific effector by *in vitro* selection will have activity in the presence of other molecules. For example, 17E deoxyribozyme was developed by *in vitro* selection for activity in the presence of Zn^{2+} . However, the enzyme showed greater activity in the presence of Pb^{2+} than Zn^{2+} . Although produced in a process looking for Zn^{2+} -related activity, 17E may be used as a sensitive and selective sensor for Pb^{2+} . To produce nucleic acid enzymes with greater selectivity, a negative selection step may be introduced.

Other polynucleotide sequences are useful, including those described in U.S. Patent Application Serial Number 09/605,558, filed June 27th, 2000, the contents of which are incorporated by reference (Lu and Liu).

5 *Aptazyme structure*

The aptazyme (Figure 2) consists of a sequence complementary to a region 3' of the cleavage site of the substrate, a DNAzyme catalytic core that is conserved, a variable region that is attached to the 3' terminus of the core (tatt (SEQ ID NO:3, indicated by "*" in Figure 2), the effector binding site (Figure 2, "aptamer motif"), and a 3' sequence that is
10 complementary to a region 5' of the substrate cleavage site. The variable region may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides longer; preferably 3-6 nucleotides long. By varying the length of the variable region, enzymatic activity and/or effector binding (selectivity and/or avidity) may be improved.

The aptazyme shown in Figure 2 is specific for adenosine. The nucleotide sequence
15 for the DNAzyme is SEQ ID NO:8, the sequence for the polynucleotide containing that aptamer is SEQ ID NO:9, and the nucleotide sequence for the substrate strand is SEQ ID NO:4.

20 *Particles tagged with polynucleotides complementary to the 3' and 5' termini of the nucleic acid enzyme substrate*

For the sensor to register the enzymatic activity, a detectable change must occur upon a change in aggregation of the particles to which the polynucleotides are attached. In addition, the composition of the particles must be such that they do not interfere with substrate cleavage. Particles may be made of metal, semiconductor and magnetic colloids;
25 ZnS, ZnO, TiO₂, AgI, AgBr, HgI₂, PbS, PbSe, ZnTe, CdTe, In₂S₃, In₂Se₃, Cd₃P₂, Cd₃As₂, InAs, and GaAs (e.g., (Mirkin et al. 2002)); and gold particles (commercially available; e.g., Amersham Biosciences; Piscataway, NJ and Nanoprobes, Inc; Yaphank, NY). Non-metal particles may also be used, such as polystyrene latex particles or latex particles containing dye.

Gold colloidal particles are preferred. Gold colloidal particles have high extinction coefficients for the bands that give rise to their intense colors. These colors vary with particle size, concentration, inter-particle distance, extent of aggregation and shape of the aggregates. For instance, hybridization of polynucleotides attached to gold particles results in an immediate color change visible to the naked eye (see, e.g., (Mirkin et al. 2002)).

Gold particles, polynucleotides or both are derivatized for the attachment of polynucleotides. For instance, polynucleotides derivatized with alkanethiols at their 3'- or 5'-termini readily attach to gold particles (Whitesides 1995). A method of attaching 3' thiol DNA to flat gold surfaces can also be used to attach polynucleotides to particles (Mucic et al. 1996). Alkanethiol-derivatized particles can be used to attach polynucleotides. Other functional groups for attaching polynucleotides to solid surfaces include phosphorothioates to attach polynucleotides to gold surfaces (Beebe and Rabke-Clemmer 1995), substituted alkylsiloxanes for binding polynucleotides to silica and glass surfaces and aminoalkylsiloxanes and mercaptoalkylsiloxanes (Grabar et al. 1995). Polynucleotides terminating in a 5'-thionucleoside or a 3'-thionucleoside may also be used for attaching polynucleotides to solid surfaces. Some methods of attaching polynucleotides are presented in Table 4.

Table 4 Systems for attaching polynucleotides to particles

System	Reference
biotin-streptavidin	(Shiau et al. 1993)
carboxylic acids on aluminum	(Allara and Nuzzo 1985)
disulfides on gold	(Nuzzo et al. 1987)
carboxylic acids on silica	(Iler 1979, Tompkins and Allara 1974)
carboxylic acids on platinum	(Timmons and Zisman 1965)
aromatic ring compounds on platinum	(Soriaga and Hubbard 1982)
silanes on silica	(Maoz and Sagiv 1987)

Preferably, the substrate is modified, for example, by extension of the 3'- and 5'-ends by a number of bases which act as "sticky ends" for facilitating annealing to the

complementary polynucleotide strand attached to the particles. Substrate modification allows complexes comprising substrate-linked particles to be formed without inhibiting the nucleic acid enzyme/substrate interaction. However, where the substrate contains regions not critical for interaction with the nucleic acid enzyme, modification may not be necessary.

5 To detect the target effector, the nucleic acid enzyme, substrate, and labeled particles are combined in the presence of a sample suspected of containing a target effector, such as adenosine, to which the enzyme is sensitive (Figure 3). In the presence of the effector, the enzyme cleaves the substrate, preventing aggregate formation. In some instances, heating the sensor system to above the melting point of the complex may be necessary. In this case, the presence of the effector allows the enzyme to cleave the substrate, preventing
10 aggregation of the complex.

Different aggregation states of the particles results in a different color. For example, a large degree of particle aggregation display colors close to blue while a small degree of particle aggregation display colors close to red. Furthermore, the amount of substrate
15 cleavage and thus the degree of aggregation depends on the concentration of the effector. A low effector concentration results in only partial substrate cleavage that produces a mixture of single particles and aggregates, allowing for semi-quantitatively or qualitative assays. The color difference can be amplified to improve sensitivity. For a quantitative measurement, the optical spectra of the assay mixture are determined. In addition to color
20 change, the formation of aggregates of the particles, or precipitation of aggregated particles may also be monitored. Color changes can be observed with the naked eye or spectroscopically. The formation of aggregates can be observed by electron microscopy or by nephelometry; and precipitation of aggregated particles can be observed with the naked eye or microscopically.

25 To facilitate the observation of a color change, the color is observed on a background of a contrasting color. When gold particles are used, the observation of a color change is facilitated by spotting a sample of the hybridization solution on a solid white surface (such as silica or alumina TLC plates, filter paper, cellulose nitrate membranes, and nylon membranes) and allowing the spot to dry. Initially, the spot retains the color of the
30 hybridization solution (which ranges from pink/red, in the absence of aggregation, to

purplish-red/purple, if there has been aggregation of gold particles). On drying, a blue spot develops if aggregation is present prior to spotting; a pink spot develops if dispersion occurred. The blue and the pink spots are stable and do not change on subsequent cooling or heating or over time. They provide a convenient permanent record of the test. No other
5 steps are necessary to observe the color change.

Alternatively, assay results may be visualized by spotting a sample onto a glass fiber filter (e.g., Borosilicate Microfiber Filter, 0.7 μ m pore size, grade FG75) for use with 13 nm gold particles. After rinsing with water, a spot comprising the aggregates is observed. Additional methods are also available for visualizing assay results (Mirkin et al. 2002).

10 The targeted effector can be detected in a variety of samples, including bodily fluids. Standards containing known amounts of the effector may be assayed along side the unknown sample, and the color changes compared. Alternatively, standard color charts, similar to those used with pH papers, may be provided.

15 *Kits*

The invention also provides kits for detecting analytes as effectors. In one embodiment, the kit comprises at least one container, the container holding at least one type of particle having polynucleotides attached thereto; a substrate; and a nucleic acid enzyme. The polynucleotides attached to the particles have a sequence complementary to the
20 sequence of at least a first and a second portion of the substrate. The first and second portions of the substrate are separated by a third portion of the substrate that is cleaved by the nucleic acid enzyme in the presence of the analyte.

A kit may also comprise at least two types of particles having polynucleotides attached thereto. A first type of particle has attached polynucleotides which have a sequence
25 complementary to the sequence of a first portion of the substrate. A second type of particle has polynucleotides attached that have a sequence complementary to the sequence of a second portion of the substrate. The first and second portions of the substrate are separated by a third portion of the substrate that is cleaved by the nucleic acid enzyme in the presence of the effector.

When a kit is supplied, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage of the active components. For example, one of more of the particles having polynucleotides attached thereto; the substrate;
5 and the nucleic acid enzyme are supplied in separate containers.

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain one of more of the reagents, or buffers that have been packaged under a neutral, non-reacting gas, such as
10 nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, *etc.*; ceramic, metal or any other material typically employed to hold similar reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may comprise foil-lined interiors, such as aluminum or an alloy. Other containers include test
15 tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to be mixed. Removable membranes may be glass, plastic, rubber, *etc.*

20 The kits may also contain other reagents and items useful for detecting the targeted effector. The reagents may include standard solutions containing known quantities of the effector, dilution and other buffers, pretreatment reagents, *etc.* Other items which may be provided as part include a backing (for visualizing aggregate break down), such as a TLC silica plate; microporous materials, syringes, pipettes, cuvettes and containers. Standard
25 charts indicating the appearance of the particles in various aggregation states, corresponding to the presence of different amounts of the effector under test, may be provided.

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audiotape, *etc.* Detailed
30 instructions may not be physically associated with the kit; instead, a user may be directed to

an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

EXAMPLES

5 The following examples are provided to illustrate the invention. Those skilled in the art can readily make insignificant variations in the compositions and methods of this invention. The examples are not meant to limit the invention in any way.

 An aptazyme designed for the directed assembly of gold particles for colorimetric detection and quantification of adenosine is herein used as a paradigm. By replacing the
10 aptamer domain that recognizes adenosine in the exemplary adenosine biosensor with other aptamer domains recognizing pre-selected effectors, colorimetric sensors for any desired effector can be easily made and used. Furthermore, by replacing the catalytic core (the 8-17 motif in this case) with other catalytic cores, similar aptazymes may be engineered.

15 Example 1 Colorimetric adenosine biosensor

Polynucleotides and reagents

 All polynucleotides were purchased from the Integrated DNA Technology Inc. (Coralville, IA). Adenosine and other nucleosides were purchased from Sigma-Aldrich (St. Louis, MO). Thirteen nm diameter gold particles were prepared and 3'- and 5'-thiol-
20 modified 12-mer DNA were attached (Storhoff et al. 1998).

Cleavage reaction

 Thirty-eight μ l of solution containing 3 μ M substrate, 6 μ M enzyme and 9 μ M regulator strands in 300 mM NaCl, 50 mM Tris-actate, pH 7.2 buffer (300 mM NTA). 2 μ l metal ion stock solution (5 mM Pb(II) and 200 mM Mg(II)) were added to initiate the
25 cleavage reaction. The final metal ion concentration was 0.25 mM Pb(II) and 10 mM Mg(II). At different times, 2 μ l aliquots were transferred to tube containing of 50 μ l gold particles with 10 μ M EDTA; the EDTA specifically chelated Pb(II) (5 μ M) in solution, even in the presence of 0.4 mM Mg(II), because the formation constant of EDTA for Pb(II) is approximately 10 orders of magnitude higher than that for Mg(II) (Sillén 1964). In the
30 presence of 0.4 mM Mg(II) only, the cleavage rate for the aptazyme is negligible. Thus, the

reaction can be considered quenched upon transferring to the gold particle solution. The 2 μ l aliquot contained 6 pmol substrate if no cleavage occurs. The cleavage can decrease the amount of substrate and that can be reflected by the degree of aggregation of the particles.

Gold particle aggregation

5 The particle solution mixed with a 2 μ l aliquot was heated to $\sim 70^\circ\text{C}$ for 3 minutes and then was allowed to cool to room temperature. The solution was assayed for the extinction property using UV-vis extinction spectroscopy or spotted onto a TLC plate.

Figure 2 shows the primary and proposed secondary structure of the three nucleic acid strands that comprise the adenosine aptazyme and two DNA-attached gold particles hybridized with the substrate strand. The two particles are designed to be positioned at the two ends of the substrate, so that two different thio-modified DNA molecules are used to attach to gold particles. One sequence has a thiol group at the 3' end of the DNA (3'-DNA_{Au}) and the other has a thiol at the 5' end (5'-DNA_{Au}). The substrate strand is a DNA/RNA chimera with a single RNA linkage that serves as the cleavage site. The strand is flanked with two 12-mer overhang that are used to hybridize with 3'-DNA_{Au} and 5'-DNA_{Au}. The catalytic core of the aptazyme is adapted from the "8-17" DNAzyme (Faulhammer and Famulok 1997, Li et al. 2000, Santoro and Joyce 1997) and has been optimized for high activity in the presence of Pb(II) (Brown et al. , Li et al. 2000). The 3' end of the DNAzyme is hybridized with the third component of the aptazyme to form an adenosine aptamer motif, obtained using SELEX process (Huizenga and Szostak 1995) and was adapted into an aptazyme (Wang et al. 2002). The presence of adenosine promotes formation of the active tertiary DNAzyme structure. This complex then promotes cleavage of the substrate strand at the single ribo-adenosine position. Without adenosine, even though the three components may interact *via* Watson-Crick base-pairing, the catalytic activity is much less than that in the presence of adenosine (Wang et al. 2002).

Figure 3 shows a schematic of the colorimetric detection of adenosine based on aptazyme-directed assembly of gold particles. In the absence of adenosine, the substrate strand in this aptazyme can be used as a linker to bring DNA modified gold particles together through hybridization to the DNA-tagged particles (reaction A). Since the color of the gold particles changes from red for separated particles to blue for aggregated particles

(Mirkin et al. 1996), upon hybridization, a blue color is observed. However, the presence of adenosine can switch on the aptazyme activity for cleaving the substrate strand (reaction B). The cleaved substrate can no longer support the gold particle aggregates. Thus the red color of separated particles is observed (reaction C). Because the rate of substrate cleavage can be modulated by the concentration of adenosine that binds to the aptamer motif, the fraction of cleavage at a set time should be dependent on the concentration of adenosine. Different ratios of the cleaved to non-cleaved substrates results in different colors, from red to blue, which reflect the adenosine concentration and can be quantified.

10 *Example 2 Demonstration of aptazyme-directed assembly of gold particles.*

Figure 4 shows the extinction spectra of gold particles in the absence (solid line) and in the presence (dash line) of the substrate strand. The shift of the extinction peak from 522 nm to 540 nm and the significant increase in extinction in the long wavelength region indicate that the presence of the substrate strand converts separated gold particles (red) to aggregated particles (blue). The melting temperature of the aggregate was determined to be 50° C in 300 mM NaCl (Figure 5). The sharp melting transition demonstrates the formation of DNA linked gold particle assembly.

Determining the optimal ratio between the substrate strand and particles, such that any decrease in the quantity of substrate induced by cleavage can be reflected in the optical properties of the particle aggregations, increases sensitivity of the assay. If the substrate is in excess, a small fraction of cleavage will be undetected. To a mixture of equal volumes (25 µl) of 5'-DNA_{Au} and 3'-DNA_{Au}, each with extinction of 2.2 at 522 nm, 1 to 9 pmol of substrate was added in the presence of excess amount of the enzyme and the regulator strands. After annealing, the extinction properties of the resulting solutions were determined by UV-vis spectroscopy. The extinction ratio between 522 nm and 700 nm was used to assay the degree of aggregation. These two wavelengths were chosen to represent the quantity of separated and aggregated particles, respectively (Figure 6). A lower ratio indicates a higher degree of aggregation. This ratio is also a measure of the color changes from red to blue. A high ratio is indicated by red and a low ratio is indicated by blue. The ratio decreases exponentially with increasing quantity of the substrate strand, showing that

the addition of substrate increases particle aggregations. When the substrate is more than 6 pmol, the degree of aggregation is no longer sensitive to a further increase in the substrate quantity. Therefore, 6 pmol of substrate are used as a starting point for forming aggregation. Any cleavage of the substrate disturbs the formation of aggregates and increases the extinction ratio.

Example 3 The kinetics of color changes of the aptazyme-gold particle sensor

In the presence of only Mg(II), the rate constant of the aptazyme was $3.5 \times 10^{-3} \text{ min}^{-1}$ with 5 mM adenosine (Wang et al. 2002), thus requiring 200 minutes for 50% substrate cleavage. The "8-17" DNAzyme is at least 3000-fold more active in the presence of Pb(II) than Mg(II) under the same conditions (Brown et al. , Li and Lu 2000, Li et al. 2000). Pb(II) was used in the system, in addition to Mg(II), to decrease detection time. Figure 7 shows the kinetics of the color changes of the aptazyme-gold particle sensor monitored by the extinction ratio between 522 nm and 700 nm in the presence of 5 mM adenosine. The kinetics data can be fit to a sigmoidal curve, thus showing that the reaction progresses to a substantial extent in 30 minutes and is complete at 60 minutes. Small errors in timing (e.g., less than 1 minute) in the sensing operation would not result in large errors in observed particle aggregations and thus color changes.

Example 4 Sensitivity and selectivity of the sensor

Thirty minutes was chosen for the assay time to determine the sensitivity and selectivity of the sensor, this time was chosen to balance both the speed and the sensitivity of the sensor. Figure 8 shows adenosine-dependent color changes; the spectra were obtained using a Hewlett-Packard 8453 spectrophotometer. Under these condition used, the sensitivity of the adenosine biosensor is 100 μM to 1 mM. Because the assay is kinetically-based, the detection range can be shifted by using different assay times.

To determine selectivity of the aptazyme-gold particle sensor, 5 mM uridine, cytosine or guanosine were used in the assay. Under these circumstances, only background signal was observed. The color difference can be conveniently monitored by spotting the resulting particles onto TLC plates. In this experiment, color progression was from blue to

purple to red, corresponding to increasing adenosine concentrations. However, assays using 5 mM of guanosine, cytidine or uridine show only blue color, the same as assays without any nucleosides added.

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CLAIMS

1. A sensor system for detecting an effector, comprising:
 - (a) a nucleic acid enzyme, comprising an aptamer comprising a binding site for the effector;
 - 5 (b) a substrate for the nucleic acid enzyme, comprising a first polynucleotide; and
 - (c) particles comprising a second polynucleotide at least partially complementary to the substrate.
- 10 2. The sensor system of claim 1, wherein the nucleic acid enzyme is DNA.
3. The sensor system of claim 1, wherein the effector is adenosine.
4. The sensor system of claim 1, wherein the effector activates the nucleic acid
15 enzyme.
5. The sensor system of claim 1, wherein the effector inhibits the nucleic acid enzyme.
- 20 6. The sensor system of claim 1, wherein the particles are gold particles.
7. The sensor system of claim 1, wherein the particles comprise a material selected from the group consisting of metal colloids, semiconductor colloids and polystyrene latex particles.
25
8. The sensor system of claim 1, wherein the effector is anthrax, an anthrax-derived molecule, small pox, a small pox-derived molecule, HIV, an HIV-derived molecule, an antibiotic or cocaine.
- 30 9. The sensor system of claim 1, wherein

the nucleic acid enzyme is selected from the group consisting of:
both SEQ ID NOs:5 and 6, both SEQ ID NOs:8 and 9, and SEQ ID NO:1;
and wherein the substrate is selected from the group consisting of: SEQ ID NO:4, SEQ ID
NO:7 and SEQ ID NO:2.

5

10. A method of detecting an effector, comprising mixing the sensor system of
claim 1 with a sample.

10

11. The method of claim 10, wherein the sensor system further comprises Mg(II).

12. The method of claim 11, wherein the sensor system further comprises Pb(II).

15

13. The method of claim 10, further comprising heating the sensor system and
sample to a melting temperature of an aggregate comprising the first and second
polynucleotide.

14. The method of claim 10, further comprising analyzing the sample for a color
change.

20

15. The method of claim 10, wherein the particles are gold particles.

16. The method of claim 10, further comprising analyzing the sample for an
aggregate comprising the particles, wherein the aggregate is a precipitate.

25

17. The method of claim 10, wherein the sample comprises a bodily fluid.

18. A sensor system for detecting an effector, comprising:

(a) a nucleic acid enzyme, comprising an aptamer comprising a binding
site for the effector;

30

(b) a substrate for the nucleic acid enzyme, comprising a first

polynucleotide;

(c) gold particles comprising a second polynucleotide at least partially complementary to the substrate; and

(d) Mg(II);

5 wherein the nucleic acid enzyme comprises DNA.

19. The sensor system of claim 18, further comprising (e) Pb(II).

20. A method of detecting an effector, comprising mixing the sensor system of
10 claim 18 with a sample.

21. A method of detecting an effector, comprising mixing together a set of ingredients comprising:

a sample;

15 a nucleic acid enzyme, comprising an aptamer comprising a binding site for the effector;

a substrate for the nucleic acid enzyme, comprising a first polynucleotide;

and

20 particles comprising a second polynucleotide that is at least partially complementary to the substrate.

22. The method of claim 21, wherein the nucleic acid enzyme is mixed with the sample before mixing the other ingredients.

25 23. The method of claim 21, wherein

the nucleic acid enzyme is selected from the group consisting of:

both SEQ ID NOs:5 and 6, both SEQ ID NOs:8 and 9, and SEQ ID NO:1;

and

30 the substrate is selected from the group consisting of: SEQ ID NO:4, SEQ ID NO:7 and SEQ ID NO:2.

24. A kit for detecting an effector, comprising
- (a) a nucleic acid enzyme, comprising an effector binding site comprising an aptamer;
- 5 (b) a substrate for the nucleic acid enzyme, comprising a first polynucleotide; and
- (c) particles comprising a second polynucleotide at least partially complementary to the substrate.
25. The kit of claim 24, wherein the substrate, particles and nucleic acid enzyme
- 10 are supplied in separate containers.
26. The kit of claim 24, wherein the substrate, particles and nucleic acid enzyme are supplied in a single container.
27. The kit of claim 24, wherein the substrate, particles and nucleic acid enzyme are supplied as an aggregate.
- 15 28. The kit of claim 24, further comprising at least one solution having a known concentration of the effector.
29. The kit of claim 24, further comprising a color chart, wherein the colors indicate a concentration of the effector.

Fig. 1

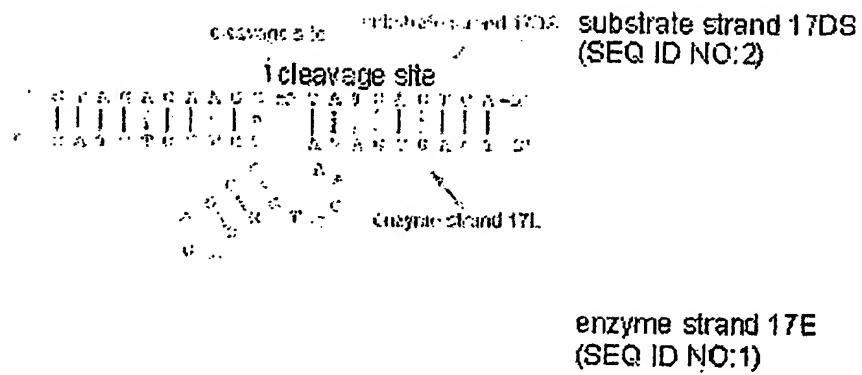
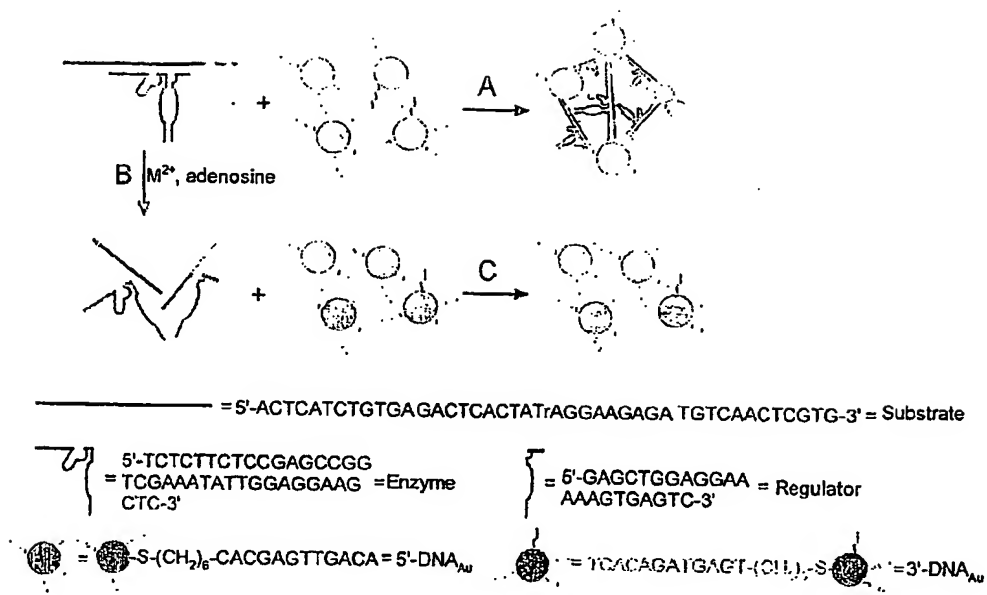


Fig. 3



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Fig. 4

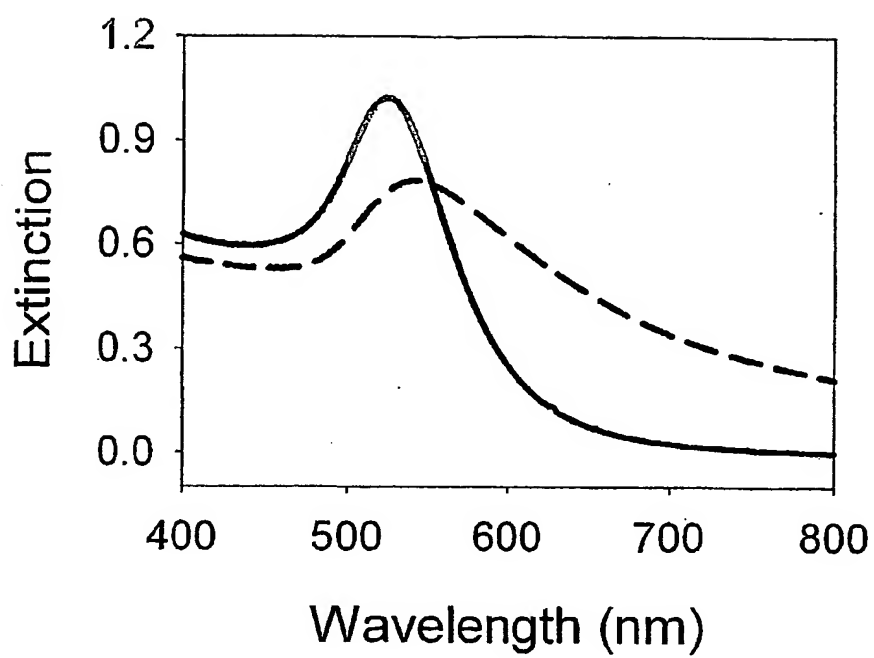
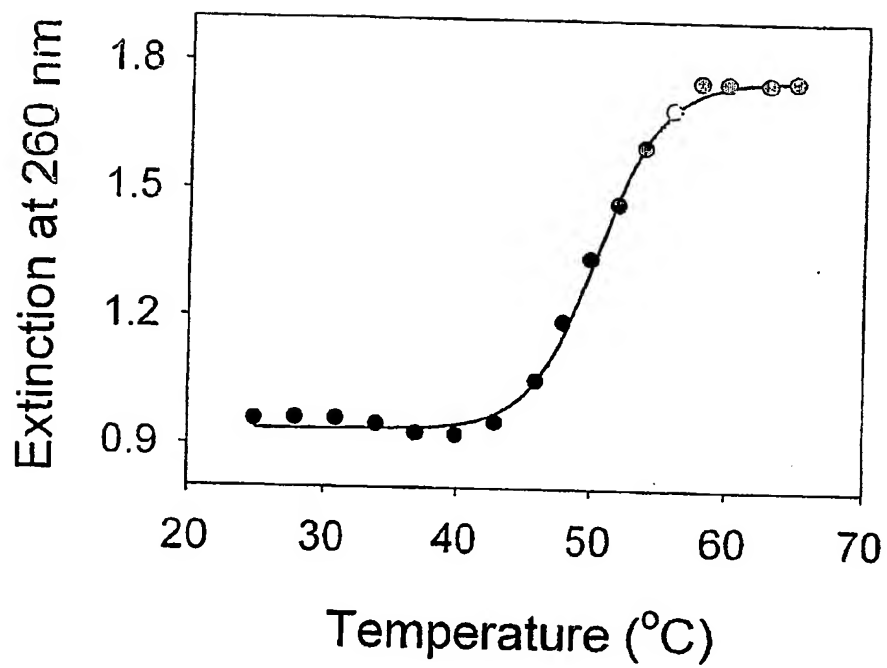


Fig. 5



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Fig. 6

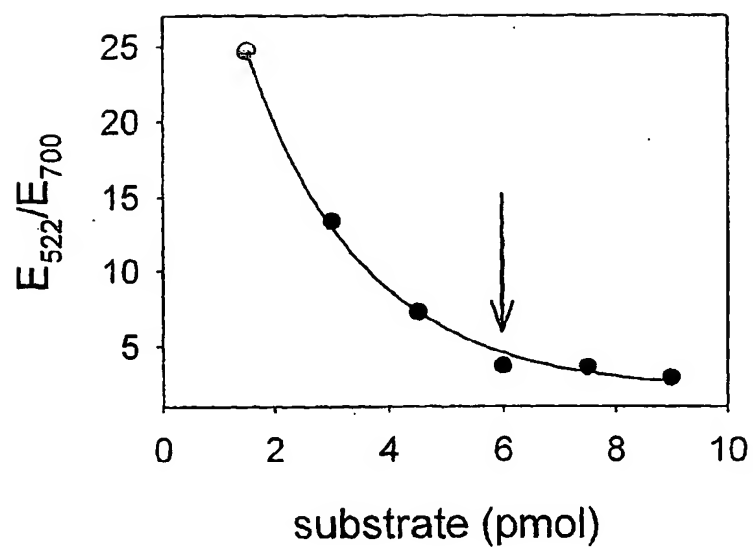
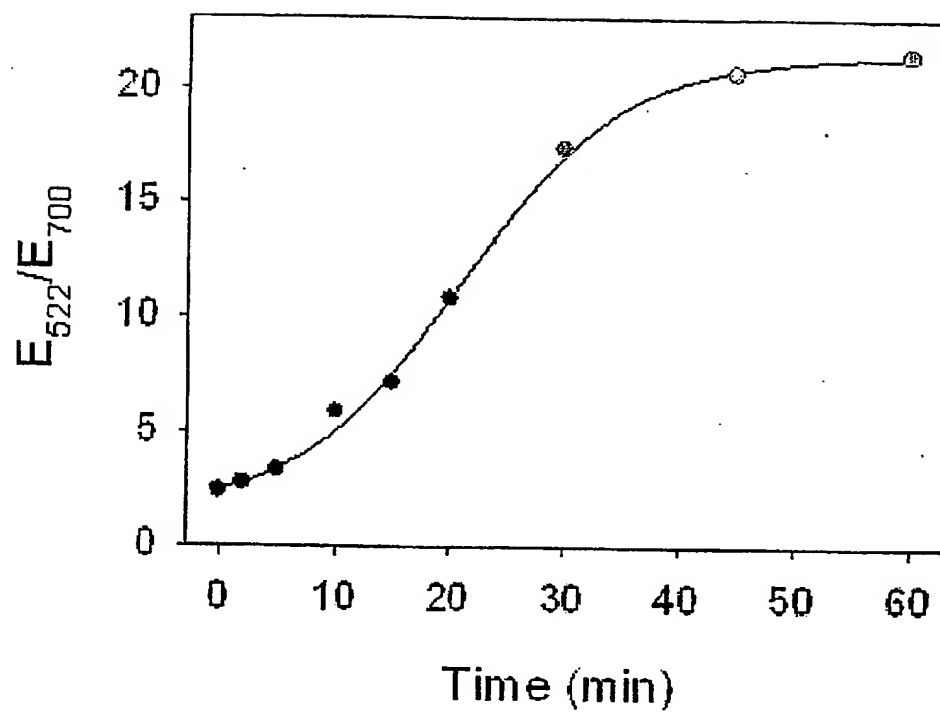
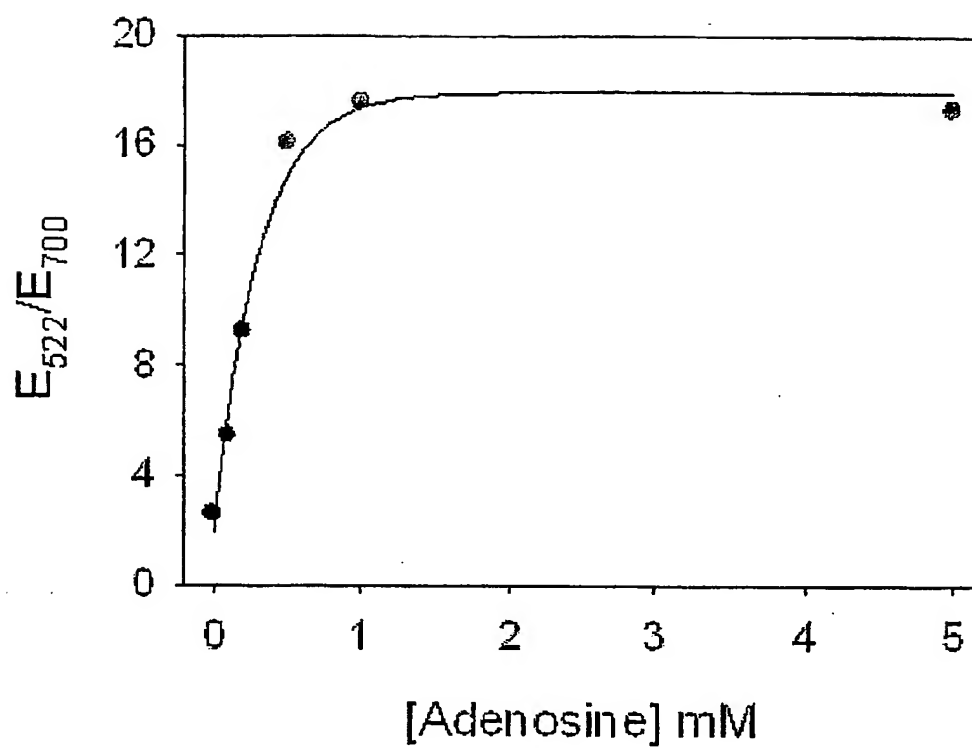


Fig. 7



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Fig. 8



SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/002946

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HESELBERTH JAY R ET AL: "Simultaneous detection of diverse analytes with an aptazyme ligase array." ANALYTICAL BIOCHEMISTRY. UNITED STATES 15 JAN 2003, vol. 312, no. 2, 15 January 2003 (2003-01-15), pages 106-112, XP002286466 ISSN: 0003-2697 the whole document</p> <p style="text-align: center;">----- -/-</p>	1-29

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

18 August 2004

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31/08/2004

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